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Treadmill running frequency on anxiety and hippocampal adenosine receptors density in adult and middle-aged rats

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ABSTRACT

Physical exercise protocols have varied widely across studies raising the question of whether there is an optimal intensity, duration and frequency that would produce maximal benefits in attenuating symptoms related to anxiety disorders. Although physical exercise causes modifications in neurotransmission systems, the involvement of neuromodulators such as adenosine has not been investigated after chronic exercise training. Anxiety-related behavior was assessed in the elevated plus-maze in adult and middle-aged rats submitted to 8 weeks of treadmill running 1, 3 or 7 days/week. The speed of running was weekly adjusted to maintain moderate intensity. The hippocampal adenosine A_1 and A_{2A} receptors densities were also assessed. Treadmill running protocol was efficient in increasing physical exercise capacity in adult and middle-aged rats. All frequencies of treadmill running equally decreased the time spent in the open arms in adult animals. Middle-aged treadmill control rats presented lower time spent in the open arms than adult treadmill control rats. However, treadmill running one day/week reversed this age effect. Adenosine A_1 receptor was not changed between groups, but treadmill running counteracted the age-related increase in adenosine A_{2A} receptors. Although treadmill running, independent from frequency, triggered anxiety in adult rats and treadmill running one day/week reversed the age-related anxiety, no consistent relationship was found with hippocampal adenosine receptors densities. Thus, our data suggest that as a complementary therapy in the management of mental disturbances, the frequency and intensity of physical exercise should be taken into account according to age. Besides, this is the first study reporting the modulation of adenosine receptors after chronic physical exercise, which could be important to prevent neurological disorders associated to increase in adenosine A_{2A} receptors.

1. Introduction

Over the past years, a wide range of studies have shown that increased physical activity or regular physical exercise promote positive effects on brain functions (Cotman and Berchtold, 2002; Dishman et al., 2006; Gillum and Obisesan, 2010; Kramer et al., 1999; Rolland et al., 2010). Otherwise, the sedentary lifestyle has been correlated with a higher risk for development of obesity, cardiovascular diseases, type 2 diabetes, osteoporosis, cancer and depression (Roberts and Barnard, 2005).

The prevalence of mental health disorders among the elderly is often unrecognized, but it has been estimated that one in four older adults presents symptoms of depression, anxiety disorders or other psychiatric disorders. Mental health disorders are frequently comorbid in older adults, occurring with a number of common chronic diseases (Fiske et al., 2009; Flint, 2005). In this scenario, the prevalence of anxiety disorders is estimated to be as high as 25% (Craske et al., 2009; Hettema et al., 2001).

Adenosine is a neuromodulator in the central nervous system (CNS), which via mainly adenosine A_1 and A_{2A} metabotropic receptors control synaptic transmission of many neurotransmitters, such as glutamate and dopamine. Adenosine A_1 receptors are expressed throughout the brain while adenosine A_{2A} is more restricted to the basal ganglia (Cunha, 2001). The physical exercise influences the central dopaminergic, noradrenergic and serotonergic systems (Meeusen and De Meirleir, 1995). In a previous study, the participation of adenosine receptors in physical activity was reported, in which caffeine increased treadmill running time and spontaneous locomotor activity on the open field task, whereas the adenosine A_1/A_{2A} receptor agonist, 5′-N-ethylcarboxamidoadenosine (NECA) decreased it (Davis et al., 2003). However, the effects of chronic physical exercise on this important neuromodulator were not investigated yet.

Pharmacological approaches and genetic deletion of adenosine A_1 and A_{2A} receptors revealed that both receptors are implicated in the

Abbreviation: VO_{2max}, maximum oxygen uptake.

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etiology of anxiety (for review see Cunha et al., 2008). The knockout mice for adenosine A_1 as well as for adenosine A_{2A} receptor display an exacerbation of anxiety-related behavior (Giménez-Llort et al., 2002; Johansson et al., 2001; Lang et al., 2003; Ledent et al., 1997). In addition, anxiogenic effects of caffeine, a recognized nonselective adenosine A_1 and A_{2A} receptor antagonist, are demonstrated in a wide range of rodent studies (File et al., 1988; Florio et al., 1998; Jain et al., 1995).

According to epidemiological studies, the physical activity can attenuate anxiety-related symptoms (Bhui and Fletcher, 2000; Dunn et al., 2001; Goodwin, 2003; Lampinen et al., 2000; Motl et al., 2004), being applied as a non-pharmacological intervention in patients, mainly in older adults (Kligman and Pepin, 1992). However, studies evaluating effects of exercise on anxiety-related behavior in rodents are more controversial, ranging from a reduction on anxiety (Greenwood et al., 2003), no effect (Pietropaolo et al., 2008), to even more anxiety after voluntary exercise (Burghardt et al., 2004; Leasure and Jones, 2008; Van Hoomissen et al., 2004).

Treadmill running is widely used by humans (Winter et al., 2007) because all variables implicated in physical training programs necessary to achieve great results can be controlled. Indeed, the frequency of the physical exercise is often controlled for maximizing the training and for avoiding negative effects of overtraining (ACSM, 1998). Nonetheless, effects of physical exercise on the anxiety-related behavior usually do not take into account the frequency of sessions and the intensity throughout training, which in turn can contribute for inconclusive results.

In this study, the effects of frequency of physical exercise were evaluated on anxiety-related behavior in adult and middle-aged rats for better understanding its effects on mental health. Considering the involvement of the adenosine receptors in anxiety disorders and its important role as a neuromodulator in the CNS, the density of adenosine A_1 and A_{2A} receptors was assessed in the hippocampus from adult and middle-aged rats trained on the treadmill running 1, 3 or 7 days/week during 8 weeks at moderate intensity. The hippocampus is a brain structure important in processes involving learning and memory, but its participation on anxiety has also been evidenced in other studies (Barkus et al., 2010; Oler et al., 2010; Sapolski et al., 1990; Xiang et al., 2011).

This schedule of exercise training (1, 3 and 7 days per week, 20 min per session at moderate intensity, for 8 weeks) was chosen to mimic exercise conditions in humans (ACSM, 1998; 2009). Thus, in this study we analyzed the effects of low and high exercise frequencies. Moreover, 8 weeks of training is considered sufficiently chronic to promote health-related adaptations.

2. Materials and methods

2.1. Animals

Adult (3 month-old) and middle-aged (14–16 month-old) male Wistar rats were used. The animals were housed into plastic cages under a light/dark cycle (lights on at 7:00 a.m.), with tap water and food ad libitum and kept at 23 °C. All procedures were carried out according to NIH Guide for Care and Use of Laboratory Animals, and Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by the ethical committee of Federal University of Rio Grande do Sul. All animals were killed by decapitation after behavioral analysis.

2.2. Treadmill exercise protocol

The treadmill exercise protocol was started from 8 to 10 a.m. Prior to the exercise training, animals were habituated in the treadmill apparatus for rat (INBRAMED TK 01, Porto Alegre, Brazil) during one week, in order to minimize novelty-induced stress. They were randomly assigned to eight experimental groups according to age (adult and middle-aged rats): treadmill controls (0); treadmill running 1 day/week (1); treadmill running 3 days/week (3), and treadmill running 7 days/week (7).

At the first day of the second week, an incremental test in the treadmill was performed to indirectly determine the maximal oxygen uptake (VO_2max) as recommended by Brooks and White (1978). This test was to determine the running speed during the training period. Briefly, animals were placed in the treadmill to run during 25 min at a low initial speed. The speed was increased by 5 m/min each 3 min up to exhaustion of the animal. Exhaustion was achieved when animals could not respond to the increase of speed. The time to fatigue (in minutes) and workload (expressed by velocity in m/min) were taken as indexes of capacity for exercise, and as a measure of VO_2max. This measure was used to control the exercise intensity during the physical training program. The incremental test was repeated in the seventh week of treadmill running training to assess the efficacy of this protocol. The intensity of the physical training protocol was adapted for each animal and it never surpassed 60–75% of the respective maximum oxygen uptake. The exercise training protocol consisted of treadmill running during 8 weeks. Each training session lasted 20 min because great improvements in physical fitness were achieved at this time (ACSM, 1998). The velocity of treadmill was weekly increased for all treadmill groups. The treadmill controls (0) were always habituated to the experimental room and handled as treadmill runners but they were not submitted to the forced running protocol (adapted from Scopel et al., 2006). The treadmill controls were placed in the treadmill off 3 days/week during 20 min. Then, animals returned to their home cages. All groups were handled every day to avoid differences in handling in the elevated plus-maze. Importantly, in the frequency of 3 days/week the animals were not submitted to the treadmill running for 3 consecutive days. There was an interval of one day between exercise days. Three days/week for control groups was chosen because this was the intermediate frequency among groups.

The velocity of running at the beginning of training was 10 m/min and 7 m/min for adult rats and middle-aged rats, respectively. This velocity was weekly increased, and at the end of training reached 20 m/min and 12 m/min for adult and middle-aged rats, respectively. This procedure was essential to keep the intensity constant in 60–75% of the maximum oxygen uptake along the 8 weeks of treadmill running training.

2.3. Elevated plus-maze

The analysis in the elevated plus-maze took place during the early light portion of the light/dark cycle. All groups were exposed to the maze on Tuesday, 48 h after the last Sunday running session for all frequencies of exercise training. This time period between the last treadmill running session and analysis in the elevated plus-maze was chosen to avoid acute exercise-related stress (Contarteze et al., 2008), which could influence the effects of treadmill running training for 8 weeks. The apparatus was made of wood covered with a layer of black formica and had four elevated arms (52 cm from the floor) 50 cm long and 10 cm wide. The arms were arranged in a cross-like disposition, with two opposite arms being enclosed (by 40 cm high walls) and two being open, having at their intersection a central platform (10 × 13.5 cm) that gave access to any of the four arms. The open arms were surrounded by a raised ledge (1 mm thick and 5 mm high). The platform was under dim light (70 lx). Each rat was placed in the central platform facing an open arm and the following observations were manually recorded during 5 min by two observers blind to the experimental groups. Each animal was tested for 5 min. Four variables were measured: (1) time spent in the open arms; (2) number of entries into the open arms; (4) number of entries into the closed arms; and (4) number of rearing. The rat was considered to have entered or spent time in an arm only when all four paws were in the respective arm.

The values obtained for time and the number of entries in the open arms are taken as measures of anxiety reduction. In addition, the number of closed arms entered was taken as an index of general activity and the number of rearing as exploratory activity (Hogg, 1996; Pellow and File, 1986).

2.4. SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) immunoblotting

Twenty-four hours after behavioral tests, rats were sacrificed by cervical displacement; the hippocampus was dissected out and immediately homogenized in 5% SDS with a protease inhibitor cocktail (Sigma, São Paulo/Brazil) and frozen at −70 °C. A sample for each set of experiments was collected for western blotting analysis. After defrost, the protein content was determined by Bicinchoninic acid assay using bovine serum albumin (BSA) as standard (Pierce, São Paulo/Brazil). Samples extracts were diluted to a final protein concentration of 2 μg/μL in SDS-PAGE buffer. Forty micrograms of the samples and prestained molecular weight standards (Bio-Rad, São Paulo/Brazil) were separated by SDS-PAGE (12% with 4% concentrating gel). After electro-transfer, the membranes were blocked with Tris-buffered saline 0.1% Tween-20 (TBS-T) containing 3% BSA for 1 h, except to adenosine A_{2A} receptors, which was blocked for 20 min. The membranes were incubated overnight for rabbit anti-adenosine A_1 receptors (1:1000, Affinity Bioreagents, São Paulo/SP, Brazil) and goat anti-adenosine A_{2A} receptors (1:500, Santa Cruz Biotechnologies, São Paulo/SP, Brazil) at 4 °C for 48 h and then reprobed for mouse anti-∝-actin (1:3000, Santa Cruz Biotechnologies, São Paulo/SP, Brazil). After primary antibody incubation, membranes were washed and incubated with horseradish-peroxidase conjugated secondary antibodies for 2 h at room temperature and developed with chemiluminescence ECL kit (Amersham, São Paulo/Brazil). The autoradiographic films were scanned and densitometric analyses were performed using public domain NIH Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Results were measured in density units using actin as a control of the protein loading.

2.5. Statistical analysis

All data were expressed as mean ± SEM and analyzed by two-way ANOVA (age × frequency as factors), followed by Fisher PLSD test when appropriate. Repeated measures ANOVA were used to assess the effects of treadmill running training on VO_{2max}. Significance was established at $P<0.05$. StatSoft Statistica 10.0 (StatSoft, Inc., USA) was the software used.

3. Results

The efficiency of our exercise training protocol was demonstrated by the performance in the second test to estimate VO_2max in adult and middle-aged rats (Fig. 1). Treadmill running increased the performance in the second test in all trained rats, which was measured by the maximal speed throughout test [F (1,113) = 4242.3, $P<0.00001$]. However, adult rats presented higher performance than middle-aged rats in all groups [F (1,113) = 383.64, $P<0.00001$]. Moreover, treadmill running 3 and 7 days/week were more efficient to increase maximum oxygen uptake estimated in both age groups [F (3, 113) = 483.35, $P<0.00001$]. The effects of age and frequency of exercise in VO_2max showed here were in accordance to human studies (ACSM, 1998). The second test was carried out in the seventh week and not in the end of the training to avoid the stress induced by test in the elevated plus-maze.

The time spent in the open arms in the elevated plus-maze was recorded for adult and middle-aged rats 48 h after the end of treadmill running training. Two-way ANOVA analysis revealed a significant interaction between frequency of running and age [F (3, 113) = 4.6889, $P=0.004$] (Fig. 2). Adult treadmill control rats (0 — white bars) spent twofold more time in the open arms than middle-aged treadmill control rats (0 — gray bars) ($P=0.0013$). For adult rats, all frequencies of treadmill running caused a similar decrease on the time spent in the open arms (45% of decrease) when compared to adult treadmill control rats [1 day/week $P=0.002876$; 3 days/week $P=0.004272$; 7 days/week $P=0.006352$] (Fig. 2, white bars). For middle-aged rats, the frequency of one day/week increased the time spent in the open arms when compared to middle-aged treadmill control rats [$P=0.0243311$] (Fig. 2, gray bars).

The closed arm entries in the elevated plus-maze were used as an index of the locomotor activity. Two-way ANOVA analysis revealed an effect of age [F (1, 113) = 5.6423, $P=0.0192$] (Fig. 3A). Two-way ANOVA analysis also revealed an effect of age in open arms entries [F (1, 113) = 4.4829, $P=0.03645$] (Fig. 3B). A significant interaction was observed in the number of entries in the open arms [F (3, 113) = 3.9729, $P=0.00986$]. Adult treadmill control rats (0 — white bars) present twofold more entries in the open arms than middle-aged treadmill control rats (0 — gray bars) ($P=0.001815$). Besides, in adult rats, treadmill running one day/week was able to decrease the number of entries in the open arms when compared to non-runners [$P=0.001236$].

As an index of exploratory activity, the number of rearing was recorded during 5 min of exploration in the elevated plus maze. Two-way ANOVA analysis revealed only a significant effect of age [F (1, 113) = 24.227, $P<0.0001$]. Thus, middle-aged rats presented

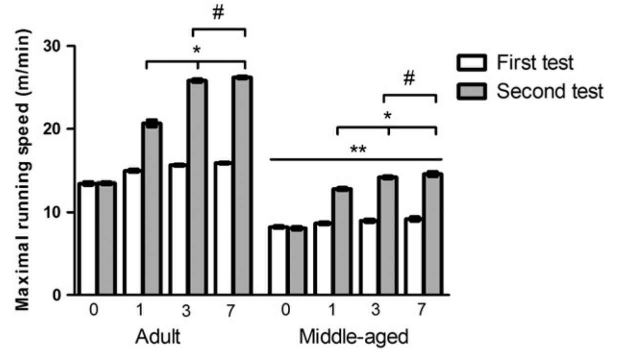

Fig. 1. Effects of treadmill running protocol on physical exercise capacity in adult and middle-aged rats. Each bar represents mean ± S.E.M of the time in the incremental test to estimated VO_2max for 16–18 adult rats or 12–14 middle-aged per group; 0 — control; 1 — one day/week; 3 — three days/week and 7 — seven days/week. Rats ran in the treadmill during 8 weeks, 20 min each session. **$P<0.05$, indicates significant effects of age (0). *$P<0.05$, indicates differences from their control counterparts. #$P<0.05$ indicates difference from their treadmill running 1 day/week counterparts (1). The data were analyzed by two-way ANOVA followed by the Fisher PLSD test.

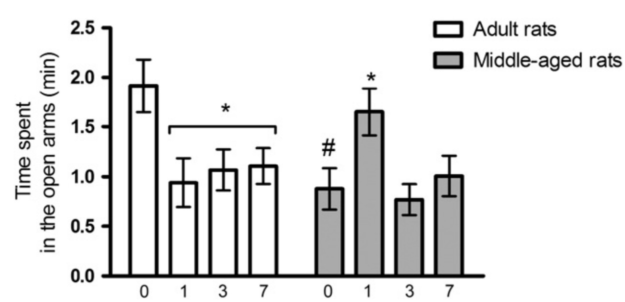

Fig. 2. Time spent in the open arms in the elevated plus-maze for adult and middle-aged rats submitted to treadmill running training. Each bar represents mean ± S.E.M of the time spent in the open arms (minutes) for 16–18 adult rats or 12–14 middle-aged per group; 0 — control; 1 — one day/week; 3 — three days/week and 7 — seven days/week. Rats ran in the treadmill during 8 weeks, 20 min each session. #$P<0.05$, indicates difference from control adult rats (0). *$P<0.05$, indicates differences from their control counterparts. The data were analyzed by two-way ANOVA followed by the Fisher PLSD test.

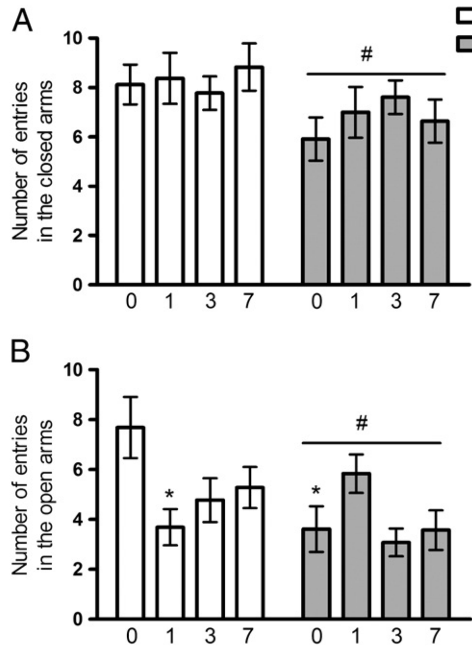

Fig. 3. Number of entries in the arms for adult and middle-aged rats in the elevated plus-maze submitted to treadmill running training. Each bar represents mean ± S.E.M of (A) the number of entries in the closed arms and (B) the number of entries in the open arms for 16–18 adult rats or 12–14 middle-aged per group; 0 — control; 1 — one day/week; 3 — three days/week and 7 — seven days/week. Rats ran in the treadmill during 8 weeks, 20 min each session. #$P<0.05$ indicates significant effects of age. *$P<0.05$, indicates differences from treadmill running control adult (0). The data were analyzed by two-way ANOVA followed by the Fisher PLSD test.

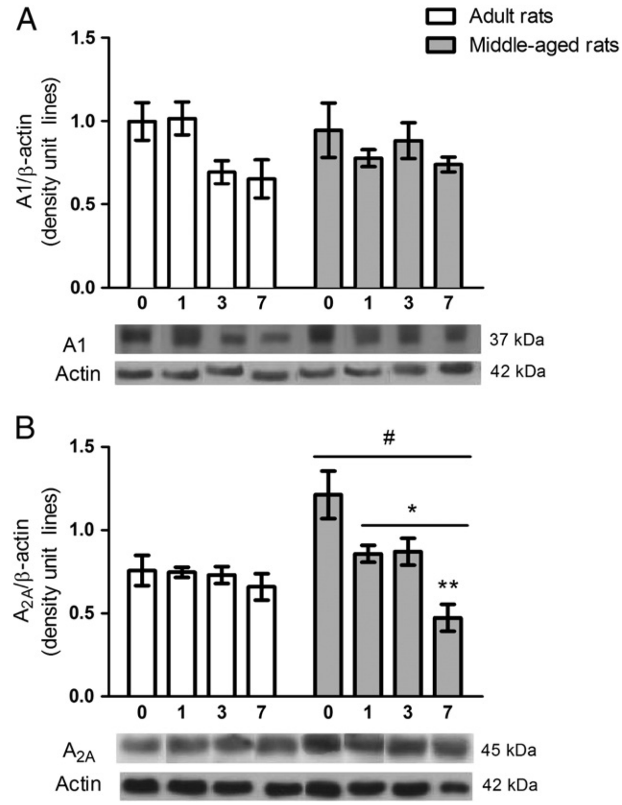

Fig. 5. Adenosine A_1 and A_{2A} receptors densities from the hippocampus of adult and middle-aged rats submitted to treadmill running. Data are expressed as means ± SEM of density unit lines. (A) Adenosine A_1 receptors densities from adult (n = 5–6 per group) and middle-aged (n = 6 per group); (B) adenosine A_{2A} receptors densities from adult (n = 5–7 per group) and middle-aged (n = 5–6 per group); 0 — control; 1 — one day/week; 3 — three days/week and 7 — seven days/week. Rats ran in the treadmill during 8 weeks, 20 min each session. #$P<0.05$, indicates significant effects of age (0). *$P<0.05$, indicates difference from control middle-aged rats (0) **$P<0.05$, indicates significant difference from all frequencies in middle-aged groups. The data were analyzed by two-way ANOVA followed by the Fisher PLSD test.

lower number of rearing when compared to adults independent on the frequency of treadmill running (Fig. 4).

The immunocontent of whole hippocampal extracts was assessed for adenosine A_1 and A_{2A} receptors (Fig. 5A and B). No differences were found in the adenosine A_1 receptors (Fig. 5A). A significant effect of age was found for adenosine A_{2A} receptors density in the hippocampus [$F(1, 39) = 4.5979$; $P = 0.0383$]. In addition, the effect of frequency [$F(3, 39) = 8.408$; $P = 0.0002$] and a significant interaction was also observed [$F(3, 39) = 5.4817$; $P = 0.00306$]. Thus, all frequencies of treadmill running significantly prevented the age-related increase in the adenosine A_{2A} receptor density, with a robust decrease for frequency of 7 days/week [$P<0.00001$] (Fig. 5B).

4. Discussion

In this study, 8 weeks of treadmill running was able to improve the physical exercise capacity, as demonstrated by the second test

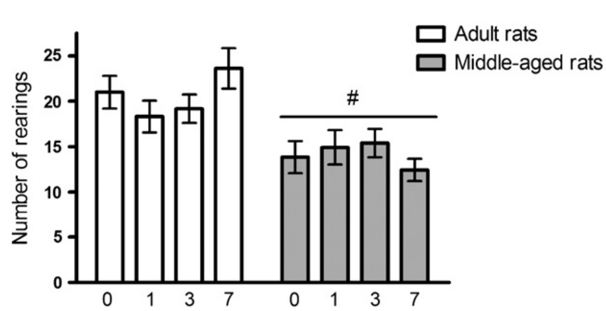

Fig. 4. Number of rearing for adult and middle-aged rats submitted to treadmill running. Each bar represents mean ± S.E.M of number of rearing for 16–18 adult rats or 12–14 middle-aged per group; 0 — control; 1 — one day/week; 3 — three days/week and 7 — seven days/week. Rats ran in the treadmill during 8 weeks, 20 min each session. #$P<0.05$ indicates significant effects of age. The data were analyzed by two-way ANOVA followed by the Fisher PLSD test.

used to estimate VO_2max in adult and middle-aged rats. Although adult rats presented higher performance than middle-aged rats in all groups, the treadmill running increased the performance in the second test in all trained rats. The frequencies of treadmill running 3 and 7 days/week presented a more robust effect on the estimated maximum oxygen uptake for both ages. The effects of age and frequency of exercise in VO_2max were similar to that found in human studies (ACSM, 1998).

In the elevated plus-maze performance, distinct effects in adult and middle-aged rats could be observed. Initially, age differences could be confirmed independent on the frequency of treadmill running. Indeed, our findings confirmed that middle-aged rats commonly exhibit a more exacerbated anxiety-like behavior than adult rats (Boguszewski and Zagrodzka, 2002; Frussa-Filho et al., 1992; Moretti et al., 2011). Besides, middle-aged rats usually present a decrease in the locomotor and exploratory activities when compared to adult ones (Agafonova et al., 2007; Stefanova et al., 2010). In this context, middle-aged animals presented less number of entries in the closed as well as in the open arms and in the number of rearing compared to adults.

The frequency of exercise preserved the habitual locomotor and exploratory activities of the animals from both ages. The frequency only modified the time spent and the number of entries in the open arms. Even though differences found in the number of entries in the closed arms (as an index of locomotor activity) can limit the interpretation of the behavior in the elevated plus maze (Lister, 1990; Pellow et al., 1985), the effects of frequency seem to be related to the anxiety-like behavior rather than locomotion for some reasons. Initially, number of entries in the arms is taken as a measure of locomotion, but current multifactorial studies have shown that this measure loads on two factors (anxiety and locomotion), whereas the number of entries in the closed arms loads exclusively (and with the highest loading) on the locomotion factor (File, 1991; Rodgers and Johnson, 1995). Since treadmill running did not affect the number of entries in the closed arms, the results found for adult rats suggest that all frequencies enhanced anxiety-like behavior. The frequency of one day/week attenuated anxiety-like behavior usually displayed by middle-aged animals.

The exercise abstinence of 48 h could explain these findings in adult rats, because anxiety is very common in trained humans during extended period of exercise abstinence (reviewed by Peluso and Guerra de Andrade, 2005). Nevertheless, this interval is necessary in order to avoid effects of fatigue caused by acute exercise as well as alterations in the locomotor activity by short intervals between the end of training and behavioral testing (Burghardt et al., 2004; Contarteze et al., 2008; Duman et al., 2008).

The impact of the physical exercise on anxiety-related behavior has reported contradictory findings in rodents. These discrepancies may be due to some factors, which include the type of exercise protocols (forced or voluntary), duration, intensity, frequency and behavioral paradigms used to assess physical exercise outcomes. Even though voluntary wheel running is largely used in studies with rodents, types of exercise will not be discussed here because this form of physical exercise does not accurately control intensity, duration and frequency of running. Furthermore, our protocol aimed to evaluate variables in the treadmill running because this type of exercise is widely used by humans.

Previous studies with behavioral analysis in the elevated plus-maze revealed that treadmill running 5 days a week during 10 weeks promoted an anxiolytic effect in adult rats (Fulk et al., 2004), whereas no effect was observed after 8 weeks of treadmill running, 5 days a week during 45 min at 20 m/min (Burghardt et al., 2004). In a recent study, adult rats that ran daily in a treadmill during 4 weeks presented normal performance in the light–dark box (Salim et al., 2010). The duration of training in these studies was similar, being considered chronic, and the interval between the end of training and the beginning of behavioral analysis was 48 h in order to avoid exercise-induced fatigue and stress (Duman et al., 2008). However, differently from other aforementioned studies in adult rats (Burghardt et al., 2004; Fulk et al., 2004), the speed of running in our protocol was weekly adjusted to maintain the intensity at 60–75% VO_2max according to the improvement of the physical capacity (see Fig. 1). It is important to emphasize that this control is crucial to maintain the intensity of running constant and promote improvement on the physical fitness (ACSM, 1998).

Our protocol revealed that only the frequency of one day a week of treadmill running was able to attenuate the anxiety-related behavior in middle-aged rats. Although the physical exercise does not interrupt the aging process, there is evidence that regular exercise can minimize detrimental effects of sedentary lifestyle and increase life expectancy by limiting the development and progression of chronic diseases and disabling conditions (ACSM, 2009; Harvey et al., 2010; Lazarov et al., 2010). The aging exacerbates anxiety-related behavior (File, 1990), and physical exercise may attenuate it (Cassilhas et al., 2010).

In this study, the protocol applied to the animals aimed to control the variables that affect outcomes of physical exercise, such as intensity, duration and frequency among training period. These variables are poorly controlled in other protocols of physical exercise used in rodents, which difficult interpretations of the findings about effects of exercise on anxiety. Moreover, this control is crucial to mimic physical exercise conditions applied in training protocols for human subjects. Here, eight weeks of moderate treadmill running for adult rats decreased the time spent in open arms, independently of exercise frequency. However, treadmill running once a week reversed the age-related anxiety. Thus, this study clearly demonstrated that the frequency of exercise affects anxiety-related behavior depending on age and reveals the importance of the amount of physical exercise necessary to promote health and fitness. The period of training (8 weeks) is considered chronic, simulates training conditions in humans and promotes health-related adaptations. The responses from acute exercise are much attributed to an increase in the levels of stress-related measurements (Winter et al., 2007).

Over the past years, the impact of different types of physical exercise on many neurotransmission systems has been investigated in rodents, and some modifications in the glutamatergic, dopaminergic and GABAergic systems were found in different brain areas (Dietrich et al., 2005; Hill et al., 2010; Real et al., 2010; Sutoo and Akiyama, 2003). The participation of adenosine receptors in physical activity was reported by administration of caffeine and the adenosine A_1/A_{2A} receptor agonist, 5′-N-ethylcarboxamidoadenosine (NECA) in rats (Davis et al., 2003). In this study, caffeine increased the running time in the treadmill and the spontaneous locomotor activity in the open field whereas the activation of A_1/A_{2A} receptor had opposite effects. Besides, caffeine was able to prevent fatigue induced by NECA in the treadmill running. It is important to consider that in our study treadmill running did not affect exploration and locomotion in the elevated plus-maze. Thus, an acute manipulation of adenosine receptors seems to influence the physical exercise performance. In our study, modifications on adenosine A_{2A} receptor density by chronic physical exercise were found. In fact, the hippocampal adenosine A_1 receptors were neither modified by frequencies of treadmill running nor by aging. Then, adenosine A_1 receptors seem not to be involved in the effects promoted by chronic treadmill running in adult and middle-aged rats. It could not be discarded that modifications might be occurring in this receptor by acute exercise.

In agreement with previous reports, the density of A_{2A} receptors increased in the hippocampus from middle-aged rats when compared to adult animals (Canas et al., 2009; Castillo et al., 2009). The most direct evidence implicating adenosine receptors in anxiety comes from studies in rodents and humans reporting the anxiogenic effects of caffeine (for review see Cunha et al., 2008; El Yacoubi et al., 2000). Currently, the anxiogenic effect of caffeine in psychiatric patients and in healthy subjects may be related to the presence of polymorphisms in the adenosine A_{2A} receptor gene (Alsene et al., 2003; Childs et al., 2008; Rogers et al., 2010; Tsai et al., 2006). Although all frequencies of treadmill running reversed the age-related increase on density of adenosine A_{2A} receptors, the same could not be observed for anxiety-related behavior. In fact, only the frequency of one day a week presented modifications on adenosine A_{2A} receptors along with attenuation in the anxiety-related behavior by aging. Even though modifications in the performance of plus maze have not been observed for frequencies of 3 and 7 days a week in middle-aged animals, physical exercise was able to prevent modifications in the adenosine A_{2A} receptors by aging. Considering that increases in the density and expression of adenosine A_{2A} receptors are associated with neurological disorders related to aging (Castillo et al., 2009; Cunha et al., 1995; Lopes et al., 1999; Meyer et al., 2007; Pagonopoulou and Angelatou, 1992), it remains to be determined whether other beneficial effects of physical exercise could be related to adenosine A_{2A} receptors.

The alterations in neuroendocrine tissues and immune responses are associated with chronic stress and similar alterations have been shown by treadmill running in adult rats (Moraska et al., 2000). The protocol applied in this study suggests that the adequate control of the intensity and frequency is indeed crucial to ensure better results and to avoid health negative effects. Besides, our data suggest that middle-aged rats had benefits of moderate exercise but only at low frequency.

Physical exercise is considered a complementary therapy in the management of mild-to-moderate mental disturbances, which include depression and anxiety (Paluska and Schwenk, 2000). Our findings contribute for the importance in controlling all variables that could generate psychological symptoms deleterious to health (Peluso and Guerra de Andrade, 2005).

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